Endogenous testosterone is not associated with the trade-off between paternal and mating effort

Eikenaar, Cas; Whitham, Megan; Komdeur, Jan; van der Velde, Marco; Moore, Ignacio T.

Published in:
Behavioral Ecology

DOI:
10.1093/beheco/arr030

Behavioral Ecology
doi:10.1093/beheco/arr030
Advance Access publication 22 March 2011

Original Article

Endogenous testosterone is not associated with the trade-off between paternal and mating effort

Cas Eikenaar,[a] Megan Whitham,[a] Jan Komdeur,[b] Marco van der Velde,[b,c] and Ignacio T. Moore[a]
[a]Department of Biological Sciences, Virginia Tech, 2125 Derring Hall, Blacksburg, VA 24061, USA, [b]Behavioural Ecology and Self-Organisation, Centre for Ecological and Evolutionary Studies, University of Groningen, PO Box 14, 9750 AA Haren, The Netherlands, and [c]Animal Ecology Group, Centre for Ecological and Evolutionary Studies, University of Groningen, PO Box 14, 9750 AA Haren, The Netherlands

Males may face a trade-off between caring for offspring and pursuing additional matings. In birds, the androgen testosterone has been suggested to be a key proximate mediator in this trade-off for several reasons. At the population level, high testosterone is typically associated with the period of intense male–male competition over females, whereas low testosterone is associated with the period of paternal care. In addition, males with experimentally elevated testosterone during provisioning feed their young at a lower rate than control males. Nearly all studies observing these patterns, however, ignore the tremendous variation in endogenous testosterone concentration that exists within a population of males, even during the same breeding stage. Because selection acts at the level of individual, this variation has to be taken into account when studying proximate mechanisms mediating the paternal and mating effort trade-off. Studying barn swallows we here show that, within males, testosterone concentrations were not higher around the fertile period of the social mate than during nestling feeding. More importantly, 30% of males showed no decrease in testosterone concentration between these 2 periods. Further, male feeding effort was not related to testosterone concentration during feeding. These results indicate that, at least in barn swallows, endogenous testosterone is not a key mediator in the trade-off between paternal effort and mating effort. Our results also stress that to understand how selection has shaped temporal testosterone profiles and action, it is crucial to study the relationship between testosterone and traits contributing to fitness at the level of the individual. *Key words:* barn swallow, endogenous testosterone, extrapair, mating effort, nestling feeding, paternal effort. *[Behav Ecol 22:601–608 (2011)]*

INTRODUCTION

Central to life-history theory is the idea that organisms are resource limited and thus that investment of resources in one trait contributing to fitness reduces investment of resources in other traits (Roff 1992; Stearns 1992). In many species, males provide both a substantial amount of care to their young and seek to enhance their fitness by trying to mate with more than one female (Ketterson and Nolan 1994). When the period of caring for the young overlaps with the occurrence of opportunities to gain additional matings, males have to trade parental effort with mating effort (Magrath and Komdeur 2003). This trade-off will be more pronounced when there are many opportunities for extrapair matings during the provisioning stage, which may occur when breeding synchrony in the population is low or when females reproduce more than once during a single breeding season. The parental effort–mating effort trade-off has received considerable attention (e.g., De Ridder et al. 2000; Trainor and Marler 2001; Van Roo et al. 2004; Lynn et al. 2005, 2009), particularly since the finding that extrapair fertilizations are common in most bird species (Griffith et al. 2002). Hormones are a likely candidate as proximate mediators of life-history trade-offs because hormones typically influence many behaviors simultaneously (Ketterson and Nolan 1992). In temperate zone birds, the androgen testosterone has been suggested to be an important mediator in the trade-off between paternal effort and mating effort (Wingfield et al. 1990; Ketterson and Nolan 1999; Hau 2007) for several reasons. Firstly, in socially monogamous birds, male testosterone concentrations are typically much higher around territory establishment and the female fertile period than during the paternal care phase (e.g., Wingfield et al. 1990; Pinxten et al. 2007; Sasvári et al. 2009). Secondly, testosterone implant experiments generally find that males with artificially elevated testosterone concentration during provisioning feed their young at a lower rate than control males (e.g., Saino and Møller 1995; De Ridder et al. 2000; Van Roo et al. 2004; Lynn et al. 2009; but see Van Duyse et al. 2000; Lynn et al. 2005). In other vertebrates, the role of testosterone in the paternal and mating effort trade-off is less clear. Similar to paternal bird species, male Puerto Rican coquí frogs (*Eleutherodactylus coqui*) provide extensive paternal care and show a strong decline in plasma testosterone concentration during the parental phase (Townsend and Moger 1987). However, testosterone implants have no effect on paternal care in this species (Townsend et al. 1991). In fishes and rodents, male testosterone, which may be elevated during care, does not always have a disruptive effect on paternal behavior, and experimental manipulation does not suggest a causal relationship between testosterone and paternal behavior (Wynne-Edwards and Timonin 2007; Neff and Knapp 2009; and references therein).

Although most bird studies do indeed suggest that testosterone is an important proximate mediator in the paternal effort–mating effort trade-off, almost all have ignored 2

Address correspondence to C. Eikenaar. E-mail: eikenaar@vt.edu.
Received 16 December 2010; revised 22 February 2011; accepted 22 February 2011.

simple, but important issues. First, seasonal testosterone profiles are typically based on the average testosterone concentrations of many different males from a population. As, within a species or population, the resolution of the paternal effort–mating effort trade-off occurs at the level of the individual (Magrath and Komdeur 2003; McGlothlin et al. 2007), it is important to confirm the population-wide trend with individual patterns of testosterone concentration. Studies doing so are scant, but Beletsky et al. (1989) have shown that in red-winged blackbirds (*Agelaius phoeniceus*), the testosterone profiles of some males do not necessarily follow the population mean. Similarly, male mandarin voles (*Microtus mandarinus*) differing in parental responsiveness to pups also differed in how testosterone concentrations changed from before pair formation to after the birth of the first litter (Smorkatcheva et al. 2010). Secondly, studies using testosterone implant experiments have generally ignored the tremendous variation in male endogenous testosterone concentration that exists within a population, even within the same breeding stage (e.g., Saino and Møller 1994; Kempenaers et al. 2008; this study). This variation in endogenous testosterone must be present for selection to act. Therefore, to fully understand the role of testosterone in the paternal effort–mating effort trade-off, we must consider the naturally occurring variation in male endogenous testosterone concentration and its relationship with mating and paternal effort (Adkins-Regan 1995; Williams 2008).

Extrapair mating effort is extremely difficult to measure (Magrath and Komdeur 2003). Measuring extrapair fertilization success may not be a good surrogate for extrapair mating effort because female extrapair mate choice is influenced by male traits other than extrapair mating effort, such as plumage coloration (Johnsen et al. 1998) or timing of molt (Green et al. 2000). A better approach would be to relate paternal effort with endogenous testosterone concentration measured in the paternal care phase. However, until now, only a handful of field studies have done so, with mixed results. In male European starlings (*Sturnus vulgaris*), testosterone concentration was negatively correlated with the proportion of time the male incubates but not correlated with male feeding rate (Pinxten et al. 2007). McGlothlin et al. (2007) found that in dark-eyed juncos (*Junco hyemalis*), male feeding rate was not related to testosterone but was negatively correlated with the magnitude of increase in testosterone after injection with gonadotropin-releasing hormone (GnRH). In tawny owls (*Strix aluco*), males with higher testosterone fed nestlings more frequently and with larger prey (Sasvári et al. 2009). In the owl study, it is unclear whether this was a direct effect of testosterone or whether this resulted from males with high testosterone having previously secured the best territories in terms of food availability. Finally, in bluegill sunfish (*Lepomis macrochirus*), neither male plasma testosterone nor 11-ketotestosterone concentration was related to paternal care (egg tending and brood defense) in the egg stage, but both androgens did show a negative relationship with paternal care (brood defense) during the fry stage (Neff and Knapp 2009).

To gain more insight into the role of testosterone as a mediator in the trade-off between paternal and mating effort, we assessed whether 1) individual testosterone concentrations are higher around the fertile period of the social mate than during feeding of nestlings and 2) there is a negative relationship between endogenous testosterone concentration and nestling feeding effort. We chose to study the North American male barn swallow (*Hirundo rustica erythrogaster*) because the seasonal testosterone profile of the population is that of a typical socially monogamous temperate bird, with testosterone concentration being high around the social mate's fertile period and low during nestling provisioning (Saino and Møller 1994). Furthermore, testosterone implants reduce feeding effort in male barn swallows (Saino and Møller 1995). Moreover, extrapair paternity is common (e.g., 31% of young, Kleven et al. 2006; 31%, this study), and most females (80% in our study population) lay a second clutch immediately after fledging of the first brood, meaning that the potential for a paternal effort–mating effort trade-off is present. Finally, as barn swallows do not defend a territory to forage, this cannot obscure a relationship between testosterone and feeding effort.

MATERIALS AND METHODS

Study species and site

Barn swallows are small (ca. 20 g) insectivorous semicolonial passerines. North American barn swallows differ phenotypically from the European race (*H. r. rustica*) in having melanin-based rust-colored feathers extending from the throat down the breast and belly to the vent (McGraw et al. 2004). Male coloration is thought to be a sexually selected character (Safran and McGraw 2004). The fieldwork in this study was carried out at 3 barns positioned within a 1 km radius on the Virginia Tech campus (lat 37°13′N, long 80°25′W, elevation ca. 700 m) from April to August 2010. The barns were occupied by 10, 15, and 24 breeding pairs of barn swallows, respectively.

Field procedures

Adults were caught passively using mist nets just previous to or during the egg-laying period and again during feeding of the nestlings. To minimize the possibility that birds were engaged in social interactions, such as territorial aggression and courtship, which may lead to short term changes in testosterone concentration (Wingfield et al. 1990), we caught birds in the early morning as they were leaving the barn (all birds caught from 5–7 AM). Immediately after capture (always within 10 min of capture), a blood sample (ca. 150 µl for males and ca. 10 µl for females) was taken from the wing vein. For male samples, the plasma was separated within 4 h of capture and frozen until hormone assaying. Female samples were stored in ethanol at room temperature for later parentage analyses. At first capture, birds received a unique combination of 2 color rings to allow identification at the nest. Length of the 2 outermost tail feathers (averaged and from here referred to as "tail length") was measured to the nearest millimeter, and mass was determined to the nearest 0.1 g. A few feathers were plucked from the belly and mounted on an index card for later objective quantification of plumage coloration with an Ocean Optics reflectance spectrophotometer (see below). Birds were sexed by the presence (females) or absence (males) of a brood patch and by observations during incubation of the nestlings. Molecular sexing confirmed all our sexing in the field. Males recaptured during the feeding period ($n = 22$) were blood sampled (ca. 150 µl) immediately after capture and then weighed, whereas recaptured females were released immediately. Again, the plasma was separated within 4 h of capture and frozen until hormone assaying. For 20 males, we obtained blood samples for testosterone analysis from both around the fertile period of the social mate and during nestling feeding. All morphological measurements were performed by C.E.

First nests were inspected every other day to determine date of clutch initiation and clutch size. Of the 49 pairs that attended a first nest, 39 produced a second clutch. To minimize disturbance of nestling feeding in late first nests, second nests were inspected only weekly. Clutch initiation dates of

second nests were estimated on finding chicks in the nest in the following way: Julian date minus 1) the estimated age (days) of the chicks, 2) 18 days (the average incubation period of first clutches in our study population), and 3) brood size minus one (incubation starts with the laying of the penultimate egg; Møller 1994). Observations during incubation and feeding served to determine which birds were associated with which nests, in other words to identify the social parents. A small (ca. 10 µl) blood sample was taken from nestlings a few days after hatching, and a tissue sample was collected if an embryo was present in eggs that failed to hatch. Samples were stored in ethanol at room temperature for later parentage analyses.

All procedures conformed to the guidelines outlined by the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were also approved by the Virginia Tech Animal Care Committee.

Behavioral observations

Observations were conducted only on first nests that were attended by a color-banded male ($n = 36$). Of these 36 nests, 29 were attended by a female that was also color banded. In these 29 nests, only the owners were observed feeding nestlings, thus, we assumed that all feedings by an unbanded female at a given nest were by the same female. Nestling feeding was filmed, and later quantified, during 3 specific periods: early (oldest nestling 6–8 days old), midway (d10–12), and late (d14–15) in the feeding period. Two of the 3 observations lasted for at least 1 h, whereas a third observation lasted for at least 2 h. The 2-h observations were always made the day before we attempted to recatch males. All observations took place in the morning (between 6 AM–12 PM) and were successful in that 96% of feeding visits could be assigned to a given male or female. Male feeding effort was quantified as the number of male feeding visits divided by the total number of feeding visits. We used a relative measure of feeding effort because this reduces the variation in male feeding effort that results from factors that we did not measure, such as insect prey densities. Compensatory behavior could have artificially increased the variation in this relative measure. For example, if females increase their feeding rate in response to low feeding rate of their mates, our relative measure of male feeding effort decreases more in comparison with absolute male feeding rate. However, pair members did not appear to compensate for each others' feeding effort (see RESULTS).

Plumage color scoring

Each male's feather sample was scored 3 times using the reflectance spectrophotometer. These scores were averaged for each sample to calculate 3 traditional indices of variation in color: hue (wavelength of maximum reflectance), brightness (mean reflectance between 320–700 nm), and saturation (sum of reflectance between 625–700 nm, divided by sum of reflectance between 320–700 nm). Prior to calculations of hue, reflectance curves were smoothed by taking the running median over 5 nm intervals.

Principal components analysis was used to collapse hue, brightness, and saturation scores into a single metric. The first principal component (PC1) explained 81% of the variation in the color scores. Ventral feathers of dark males have higher hue (eigenvector = 0.54) and are more saturated (eigenvector = 0.60) and less bright (eigenvector = −0.59) than those of drab males. In the data analyses, we used this PC1 of color, and in the remainder of the text, we refer to this measure as "ventral plumage color."

Hormone analysis

All blood plasma samples from males were analyzed for testosterone in duplicate by radioimmunoassay following the procedures of Wingfield et al. (1991) and Moore et al. (2002). Sample volumes ranged from 14–77 µl (mean: 55 µl). We performed a direct assay, thus measuring total androgen (testosterone + 5α-dihydrotestosterone). The limit of detection depended on individual plasma volumes and was approximately 0.14 ng/ml. The samples were run in a single assay with a mean extraction efficiency of 77% and an intra-assay variation of 12.8%. The testosterone antibody used was T-3003s (Fitzgerald: Catalog # WLI-T3003s, New catalog #20R-TR018W). Typically in birds the concentration of endogenous 5α-dihydrotestosterone is extremely low and often undetectable in individual blood samples (e.g., undetectable in 44% of 92 male barn swallow samples, Saino and Møller 1994), thus endogenous testosterone concentrations will be extremely similar to endogenous total androgen concentrations. We therefore hereafter refer to total androgen as testosterone.

Molecular sexing and parentage analysis

DNA was extracted from blood and tissue samples using a chelex extraction method (Walsh et al. 1991) or salt extraction method (Richardson et al. 2001). Sex of all DNA samples was determined following Griffiths et al. (1998) and/or Fridolfsson and Ellegren (1999). To exclude and assign paternity, parents and chicks were genotyped for 4 microsatellite loci: Esc6 (Hanotte et al. 1994), Pocc6 (Bensch et al. 1997), and Hru5 and Hru6 (Primmer et al. 1995). Polymerase chain reactions (PCRs) were carried out in 10 µl volume using 20–50 ng of template DNA, a QIAGEN Multiplex PCR Kit and manufacturers protocol. Final primer concentrations were 0.2 µM for Pocc6 and Hru5 and 0.1 µM for Esc6 and Hru6. Microsatellite loci Esc6 and Pocc6 and loci Hru5 and Hru6 were amplified simultaneously using the following PCR program: 15 min 95 °C, 35 cycles of 94 °C for 30 s, 50 °C (Esc6, Pocc6) or 58 °C (Hru5, Hru6) for 90 s, and 72 °C for 60 s, followed by 60 °C for 30 min. Fluorescently labeled PCR products were separated on an AB3730 DNA analyzer. Subsequently, allele lengths were determined using Genemapper 4.0 software. Using Cervus 3.0 (Kalinowski et al. 2007), mean exclusion probability of the 4 markers was calculated to be 0.9915 for the first parent and 0.9989 when the identity of both social parents was known. Paternity of the social male was excluded if there was at least one mismatch between the genotypes of the social father and nestling, and those nestlings were regarded as extrapair young (EPY).

Data analyses

We used a paired samples *t*-test to compare individual males' testosterone concentrations measured during the social mate's fertile period and the nestling feeding period because the differences between pairs of testosterone concentrations were normally distributed. Repeatability (r) of male testosterone concentrations was calculated after Lessells and Boag (1987). Individual testosterone concentration may vary as a result of differences in extrinsic factors at the time of sampling, such as sexual state (fertile or not) of the social mate (Wingfield et al. 1990), time of day (Balthazart 1976), or breeding density (Sasvári et al. 2009). At the start of the breeding season, we caught and blood-sampled males during 6 different mornings, the first being 30 April and the last being 12 May. Treating all the resulting testosterone values as having come from the same substage of the breeding cycle (in

the text referred to as "around the fertile period of the social mate") needs justification. To that purpose, we performed a multilevel generalized linear mixed model (GLMM) with a normal error structure using MlwiN 2.0 (Rasbash et al. 2004) to analyze the effect of the following extrinsic factors on testosterone concentration: day of capture expressed as the number of days before or after the social mate's clutch initiation, time of capture, ambient temperature at capture, opportunities for extrapair activity expressed as the number of fertile females present per male on the day of capture, and breeding density expressed as the number of active nests within 6 m of the own nest. The female fertile period was assumed to last from 6 days before clutch initiation until the day the penultimate egg was laid (Birkhead and Møller 1992). Extrapair opportunities were calculated at the scale of the barn because in our study population, 1) males never sired EPY outside their own barn, and 2) within the barns, extrapair males often sired young in nests situated a long way from their own nest (Eikenaar C, unpublished data). Breeding density was measured within 6 m as this was the average distance males had to fly through the barns, and could thus interact with other swallows, to reach their nests. Model selection was based on backward elimination of nonsignificant terms. Reported values are as in the step prior to elimination from the model or as in the final model. By entering barn as a random factor, we accounted for the nonindependence of males breeding in the same barn.

We compared behavioral and morphological variables (tail length, mass, and ventral plumage color) between specific sets of resampled males using GLMMs in MlwiN 2.0. Cuckoldry rate, that is, the proportion of EPY in a male's own brood, was analyzed using a binomial error distribution with a logit link function. By entering the number of offspring in a brood as the denominator, we accounted for the variation in brood size (1–6 nestlings). Because extrapair fertilization success was highly skewed toward males that did not gain EPY, this variable was also analyzed using a binomial error distribution (yes/no EPY) with a logit link function. The denominator was set to 1. Male feeding effort, the total number of offspring produced, and morphological variables were analyzed using a random error distribution. Barn was entered as a random factor in all analyses.

To determine whether male feeding effort (dependent variable) was related to male testosterone concentration during feeding, we performed multilevel GLMMs with a normal error distribution, using MlwiN 2.0. By entering barn and male as random factors, we accounted for the nonindependence of observations from the same barn and multiple observations of the same male. Testosterone concentrations were ln-transformed prior to analyses. We ran 2 analyses: one in which all 3 feeding observation periods of the 22 recaptured males were used ("larger model"), and one using only the 2-h observations made the day before males were recaptured and blood sampled ("smaller model"). In the larger model, the following potentially confounding variables of the relationship between testosterone and feeding were entered into the model: male body mass at recapture, male ventral plumage color, male tail length, cuckoldry rate, that is, the proportion of EPY in the brood, and opportunities for extrapair activity expressed as the number of fertile females present in the barn per male on the day of the observation. To avoid over parameterization, only those variables that showed a significant relationship with male feeding effort in the larger model were entered in the smaller model together with male testosterone concentration. Model selection was based on backward elimination of nonsignificant terms. Reported values are as in the step prior to elimination from the model or as in the final model. Probabilities are 2-tailed in all tests.

RESULTS

Temporal variation in male testosterone concentration

Extrinsic factors

Around the fertile period of the social mate, the variation in male testosterone concentration was not explained by variation in any of the extrinsic factors in our model (Table 1). The results did not differ in any significant way when including only the 20 males that were used for the within-male comparison of testosterone across the breeding season. This indicates that all males were sampled in the same stage of the breeding cycle and justifies the within-male comparison of these testosterone concentrations with concentrations during feeding of the nestling.

Within-male variation

Figure 1 shows the testosterone concentrations of the 20 males that were caught and sampled twice during the breeding cycle of their first nest (day 0 is the day the first egg was laid in each individual nest). Thus, values in the left part of the graph are testosterone concentrations around the fertile period of the social mate, henceforward called the "mating" testosterone. Values in the right part are testosterone concentration during nestling feeding, henceforward called "feeding" testosterone. There was a trend for individual males' mating testosterone concentration to be higher than their testosterone concentration when feeding young (paired samples *t*-test: $t = -2.06$, $P = 0.054$ and $n = 20$). Interestingly, there was a lot of variation among males in this pattern. Five of the 20 males had higher testosterone concentrations during feeding than during the mating period, and 1 male had equal testosterone concentrations in the 2 periods (these 6 males are indicated in Figure 1 with letters A–F). Accordingly, the repeatability of male testosterone concentration was low ($r = 0.07$, $F_{19,20} = 1.16$ and $P = 0.37$). Males that did and males that did not show a decrease in testosterone between the 2 periods did not differ in morphology (tail length, mass, and ventral plumage color) or feeding effort (all $P > 0.72$, $n = 20$). Males that did not decrease testosterone had a higher cuckoldry rate in their first nests than males that did decrease testosterone ($\beta \pm$ standard error [SE] $= 1.29 \pm 0.53$, $\chi^2 = 5.99$, $P = 0.014$, and $n = 20$). Cuckoldry rates in second nests, however, did not differ between the 2 groups of males nor did the number of EPY they sired in second nests or the total number of offspring produced in the breeding season (all $P > 0.2$, $n = 20$).

The lack of a significant difference in mating and feeding testosterone does not appear to be due to the inclusion of measurements taken relatively early and late in the mating

Table 1

Relationships between individual testosterone concentration in male barn swallows and extrinsic factors ($n = 36$)

Extrinsic factors	$\beta \pm$ SE	χ^2	df	*P*
Day of capture	-0.042 ± 0.032	1.68	1	0.19
Time of capture	-2.117 ± 12.334	0.03	1	0.86
Ambient temperature at capture	0.005 ± 0.017	0.08	1	0.78
Breeding density	0.044 ± 0.111	0.16	1	0.69
Opportunities for extrapair activity	0.026 ± 0.060	0.18	1	0.67

Day of capture was expressed as the number of days before or after the social mate's clutch initiation, breeding density as the number of active nests within 6 m of the own nest, and opportunities for extrapair activity as the number of fertile females present in the barn per male on the day of capture. df, degrees of freedom. Summaries derived from the mixed modeling procedure in MLwiN.

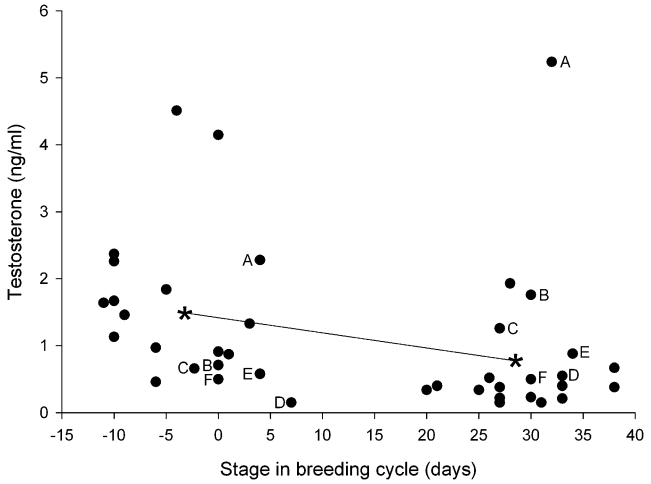

Figure 1
Testosterone concentrations of 20 male barn swallows in relation to the stage in the breeding cycle of their first nest. Each males' testosterone concentration is depicted twice; once around the fertile period of his social mate (left part of the plot) and again during feeding of the nestlings (right part of the plot). Letters indicate the testosterone concentrations of the 6 males that showed no decrease between the 2 periods. The 2 stars connected by the solid line indicate the mean testosterone concentration of the 20 males around the fertile period of their social mates (1.52 ng/ml) and during feeding of the nestlings (0.82 ng/ml). Day 0 is the day the social mate laid the first egg.

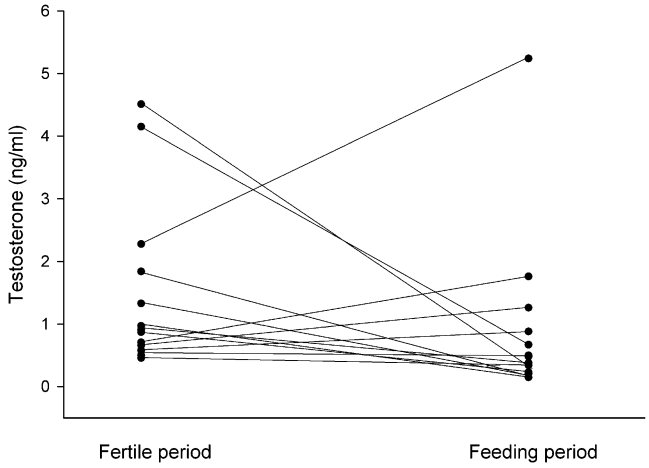

Figure 2
Testosterone concentrations of 13 individual male barn swallows that were sampled both in the fertile period of their social mate and when feeding their nestlings. Pairs of testosterone concentrations of each male are connected by a solid line.

period. When restricting the analysis to only those males whose mating testosterone was measured in the fertile period of their social mate, there was no difference between mating and feeding testosterone (paired samples *t*-test: $t = -1.12$, $P = 0.28$, and $n = 13$; Figure 2).

Testosterone and male feeding effort

Pair members did not appear to compensate for each others' feeding effort; there was a very strong positive correlation between the absolute number of male and female feeding visits per observation ($P \leq 0.001$ at all 3 [early, halfway, and late] stages in the feeding period).

Table 2 presents the relationships between male feeding effort (the number of male feeding visits divided by the total number of feeding visits in an observation) and several male phenotypic traits and social variables. Males contributed less to feeding of the nestlings when there were more fertile females present per male in the barn (Figure 3). Male feeding effort was also negatively related with male body mass. In the smaller model, male feeding effort was negatively related with male body mass ($\beta \pm SE = -0.128 \pm 0.034$, $\chi^2 = 14.08$, $P < 0.001$, and $n = 22$) but was not related with the number of fertile females present per male in the barn ($\chi^2 = 0.001$, $P = 0.97$, and $n = 22$). Male feeding effort was not related to male testosterone concentration, neither in the larger model (Table 2) nor in the smaller model ($\chi^2 = 0.12$, $P = 0.73$, and $n = 22$; Figure 4). Replacing relative male feeding effort with absolute male feeding effort (number of feeds per hour) in the analyses did not change the results in any significant way.

DISCUSSION

In the analysis including 3 observations of each male's feeding effort, males contributed less to feeding of the nestlings when there were more fertile females present in the barn (Table 2). This strongly suggests that male barn swallows in our study population traded paternal effort with extrapair mating effort. We, however, found no evidence for a role of testosterone as a proximate mediator in this trade-off. First, within males, testosterone concentrations tended to be, but were not, significantly higher around the fertile period of the social mate than during feeding of the nestlings. More importantly, we also found that a considerable proportion (30%) of the males showed no decrease but actually an "increase" in testosterone from around the fertile period of the social mate to the parental care phase. Second, individual male feeding effort was not related to individual testosterone concentration during feeding. The lack of a relationship was not the result of testosterone concentrations being very low in the period of nestling feeding (see Figure 1).

Although several papers report to have blood-sampled free-living males more than once (e.g., Saino and Møller 1994; Pinxten et al. 2007), typically for each male, only a single testosterone concentration is selected and used for statistical

Table 2
Relationships between male barn swallows feeding effort (the number of male feeding visits divided by the total number of feeding visits in an observation) and testosterone concentration, male body mass, ventral plumage color and tail length, cuckoldry rate, and opportunities for extrapair activity ($n = 65$)

Extrinsic factors	$\beta \pm SE$	χ^2	df	P
Testosterone	0.01 ± 0.017	1.03	1	0.31
Body mass	-0.054 ± 0.019	7.98	1	0.005
Plumage color	-0.021 ± 0.012	2.86	1	0.09
Tail length	-0.003 ± 0.002	1.46	1	0.23
Cuckoldry rate	-0.046 ± 0.034	1.87	1	0.17
Opportunities for extrapair activity	-0.213 ± 0.075	8.01	1	0.004

Cuckoldry rate was expressed as the proportion of EPY in the brood, and opportunities for extrapair activity as the number of fertile females present in the barn per male on the day of the observation. Summaries derived from the mixed modeling procedure in MLwiN. df, degrees of freedom.

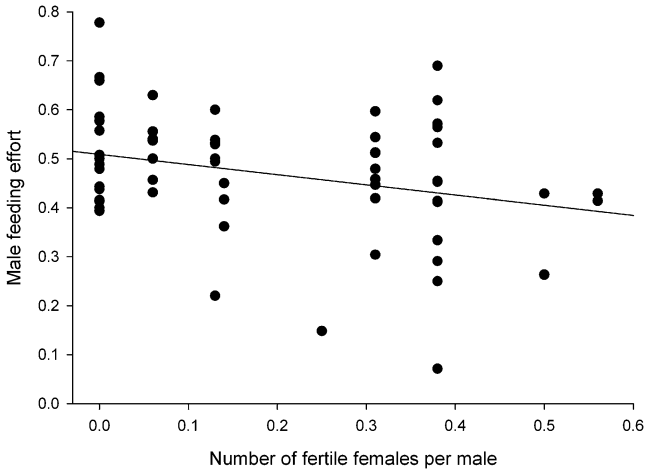

Figure 3
Male barn swallow feeding effort in relation to the number of fertile females present per male in the barn. Plotted are the results from 3 observations on feeding effort of 22 males. The 3 observations were conducted early (oldest nestling 6–8 days old), midway (d10–12), and late (d14–15) in the feeding period of each male. The regression line indicates the negative relationship between male feeding effort and the number of fertile females present.

analysis and graphical presentation. Our study, however, clearly shows that seasonal testosterone profiles can differ dramatically among males. This finding is in agreement with the handful of previous studies that also report repeated testosterone measurements of the same males in different stages of the breeding cycle. Beletsky et al. (1989) found that, in red-winged blackbirds, not all males' testosterone profiles rigidly follow the population mean. Jawor et al. (2006) found that, in dark-eyed juncos, individual male testosterone concentrations are not repeatable between different stages of breeding. Magee et al. (2006) showed that individual male testosterone concentration of bluegill sunfish can either increase or decrease from the egg stage to the fry stage. Taken together, our study and these previous findings indicate that linking

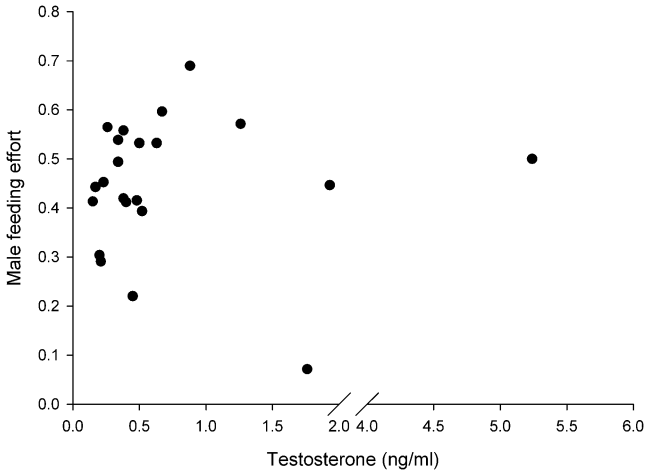

Figure 4
Male barn swallow feeding effort in relation to male testosterone concentration during nestling feeding. Feeding effort was calculated as the number of male feeding visits divided by the total number of feeding visits observed in a 2-h observation made the day before testosterone samples were taken. $N = 22$.

the occurrence of certain behaviors to population averages of hormone levels can be misleading. To better understand the proximate role of hormones in life-history trade-offs, studies should not only be directed at population averages but also at individual hormone profiles.

By determining individual hormone profiles, we discovered 2 contrasting patterns; some males did, and other males did not decrease testosterone from around the fertile period of the social mate to the parental care phase (Figures 1 and 2). Interestingly, males that did not decrease testosterone between the 2 periods had a higher cuckoldry rate in their first nests than males that did decrease testosterone. It is intuitively appealing to suggest this difference exists because cuckolded males elevated their testosterone in order to be better able to protect their paternity in the second nest or to gain more EPY themselves in second nests of other males. Another explanation may be that males that lost much of their parentage probably had many encounters with rival males during the period of mate guarding, which resulted in elevated testosterone during feeding. Neither of these explanations, however, makes much sense in the light of the findings of the current and a previous study, we conducted on the same population. The first explanation implies that males have some idea of the degree of infidelity of their mate. This seems unlikely, as the proportion of EPY in a nest did not affect male feeding effort (Table 2). Also, males that that did not decrease testosterone were not better at protecting their paternity in their second nest nor did they achieve more EPY in other males' second nests than males that did decrease testosterone (see RESULTS). This was to be expectedbecause another study in this population found that male testosterone concentration did not predict within or extrapair fertilization success (Eikenaar C, Whitham M, Moore IT, unpublished data). Regarding the second explanation, a high encounter rate with rival males should have resulted in elevated testosterone concentrations in the period of mate guarding, which is tightly linked to the period that the female is fertile. Clearly, this did not happen (see males A–F in Figure 1).

In bluegill sunfish, elevated male testosterone during care appears to mediate renesting potential rather than paternal behavior (Magee et al. 2006; Neff and Knapp 2009). However, in our study, elevated testosterone during care is unlikely to be associated with renesting potential because nearly all males renested after their first brood fledged. Why, in our study, male seasonal testosterone profiles varied dramatically remains to be answered.

Our finding that individual male feeding effort was not related to individual testosterone concentration during feeding contrasts with the observation that in European barn swallows testosterone implants reduce male feeding effort (Saino and Møller 1995). Similarly, in dark-eyed juncos and European starlings, male feeding effort was reduced in testosterone implanted males (Ketterson et al. 1992; De Ridder et al. 2000) but not correlated with endogenous testosterone during nestling feeding (McGlothlin et al. 2007; Pinxten et al. 2007). In the dark-eyed juncos, male feeding rate was, however, negatively correlated with the magnitude of increase in testosterone after GnRH injection (McGlothlin et al. 2007). Discrepancies in the relationships between paternal behavior and either endogenous or exogenous testosterone are not limited to birds. Similar to paternal bird species, male Puerto Rican coquí frogs provide extensive paternal care and show a strong decline in plasma testosterone concentration during the parental phase (Townsend and Moger 1987). However, testosterone implants have no effect on paternal care in this species (Townsend et al. 1991). In California mice (*Peromyscus californicus*), endogenous testosterone concentrations are not different between parental (fathers) and nonparental (virgin)

males (Gubernick and Nelson 1989). However, Trainor and Marler (2001) showed that, in the same species, testosterone implants increase paternal care in castrated males. Given the inconsistency of results from observation and experiment, it would be very interesting to collect observational data on other species in which experimental testosterone implants have been shown to reduce male feeding effort. Another incentive to collect observational data is that this may be more informative regarding the role of testosterone as a proximate mediator in the paternal effort–mating effort trade-off than testosterone implant experiments. The reason for this is that a classic testosterone implant experiment does not take into account the enormous variation in testosterone concentration that exists among males, even within the same breeding stage. In other words, they fail to study the (proximate) resolution of the trade-off at the level of the individual as typically the (feeding) behavior of a testosterone-enhanced group of males is compared with that of a control group. Taking into account individual variation in testosterone concentration is important as selection acts at the level of the individual. Therefore, to understand how selection has shaped temporal testosterone profiles, it is crucial to study the relationship between testosterone and traits contributing to fitness at the level of the individual (Williams 2008). Moreover, testosterone implants usually raise endogenous concentrations as high as the population maximum observed around territory defense and egg laying (e.g., Saino and Møller 1995; Van Roo et al. 2004; Lynn et al. 2009). This means that for the large majority of testosterone implanted males, their feeding testosterone concentration is many times higher than their peak endogenous concentration. Consequently, it may be difficult to interpret the behavior associated with these artificially high testosterone concentrations (Adkins-Regan 1995). To complement observational data, a better but logistically more challenging implant experiment would be to quantify the (feeding) behavior of the same individual before and after a treatment in which testosterone is increased to each individual's peak concentration. It is worth noting that one of the few studies to quantify feeding effort of individual males before and after a treatment did not observe a difference in feeding effort between testosterone and control-implanted individuals (Van Duyse et al. 2000).

If not testosterone, what does mediate the paternal effort–mating effort trade-off? Most likely a multitude of intrinsic and extrinsic factors jointly influence the resolution of this trade-off by affecting the benefits of either mating or paternal effort (Magrath and Komdeur 2003). Our results, for example, indicate that an increase in opportunities to pursue extrapair matings negatively affects paternal effort, probably because the benefits of mating effort are increased. Food availability may also affect male effort to attract additional mates. For example, in some biparental burying beetles (*Nicrophorus* spp.), males try to attract additional females when the carcass they buried to supply food for their developing young is large enough to support more than the brood of 1 female (Trumbo and Eggert 1994). As a last example, the energetic requirements of a brood could also influence the paternal effort–mating effort trade-off. In species, where male care is critical to the survival of nestlings (essential care hypothesis; e.g., Lynn et al. 2005), the benefits of paternal effort are expected to greatly outweigh the benefits of mating effort. Consequently, males of these species may invest little of their resources in pursuing additional matings. Future (experimental) studies into the paternal effort–mating effort trade-off should take into account the idea that it is unlikely a single factor that determines the resolution of this trade-off.

In conclusion, our study indicates that, at least in North American barn swallows, endogenous testosterone is not a key mediator in the paternal effort–mating effort trade-off. Our findings stress that, to understand how selection has shaped temporal testosterone profiles, it is crucial to study the relationship between testosterone and traits contributing to fitness at the level of the individual. Moreover, our observation that a considerable proportion of males showed no decrease in testosterone concentration from around the fertile period of the social mate to the feeding period warrants an increased effort to resample males in different stages of the breeding cycle.

FUNDING

Grants from National Science Foundation awarded to Ignacio Moore (IOS 0545735), from Nederlandse Organisatie voor Wetenschappelijk Onderzoek (VICI 86503003) awarded to J.K. and from Stichting Nicolaas Mulerius Fonds and Dobberke Stichting, both allocated to C.E.

We are grateful to Dave Linker, Celeste Crisman, Shane Brannock, and their colleagues for allowing us to work at the farms on the Virginia Tech campus. We thank Ashley Grapes and Alex Tallant for help in the field and Karen Bouwman for assistance with statistical analyses. Oddmund Kleven kindly provided advice on the microsatellite genotyping of the North American barn swallow.

REFERENCES

Adkins-Regan E. 1995. Hormones and animal social behavior. Princeton (NJ): Princeton University Press.

Balthazart J. 1976. Daily variations of behavioral activities and plasma testosterone levels in the domestic duck. J Zool. 180:155–173.

Beletsky LD, Orians GH, Wingfield JC. 1989. Relationships of steroid hormones and polygyny to territorial status, breeding experience, and reproductive success in male red-winged blackbirds. Auk. 106: 107–117.

Bensch S, Price T, Kohn J. 1997. Isolation and characterization of microsatellite loci in a *Phylloscopus* warbler. Mol Ecol. 6:91–92.

Birkhead TR, Møller AP. 1992. Sperm competition in birds, evolutionary causes and consequences. London: Academic Press.

De Ridder E, Pinxten R, Eens M. 2000. Experimental evidence of a testosterone induced shift from parental to mating behavior in a facultatively polygynous songbird. Behav Ecol Sociobiol. 49:24–30.

Fridolfsson AK, Ellegren H. 1999. A simple and universal method for molecular sexing of non-ratite birds. J Avian Biol. 30:116–121.

Green DJ, Osmond HL, Double MC, Cockburn O. 2000. Display rate by male fairy wrens (*Malurus cyaneus*) during the fertile period of females has little influence on extra-pair mate choice. Behav Ecol Sociobiol. 48:438–446.

Griffith SC, Owens IPF, Thuman KA. 2002. Extra pair paternity in birds: a review of interspecific variation and adaptive function. Mol Ecol. 11:2195–2212.

Griffiths R, Double MC, Orr K, Dawson RJ. 1998. A DNA test to sex most birds. Mol Ecol. 7:1071–1075.

Gubernick DJ, Nelson RJ. 1989. Prolactin and paternal behavior in the biparental California mouse, *Peromyscus californicus*. Horm Behav. 23: 203–210.

Hanotte O, Zanon C, Pugh A, Greig C, Dixon A, Burke T. 1994. Isolation and characterization of microsatellite loci in a passerine bird: the reed bunting *Emberiza schoeniclus*. Mol Ecol. 3:529–530.

Hau M. 2007. Regulation of male traits by testosterone: implications for the evolution of vertebrate life histories. Bioessays. 29:133–144.

Jawor JM, McGlothlin JW, Casto JM, Greives TJ, Snajdr EA, Bentley GE, Ketterson ED. 2006. Seasonal and individual variation in response to GnRH challenge in male dark-eyed juncos (*Junco hyemalis*). Gen Comp Endocrinol. 149:182–189.

Johnsen A, Andersson S, Örnborg J, Lifjeld JT. 1998. Ultraviolet plumage ornamentation affects social mate choice and sperm competition in bluethroats (Aves: *Luscinia s. svecica*): a field experiment. Proc R Soc Lond B Biol Sci. 265:1313–1318.

Kalinowski ST, Taper ML, Marshall TC. 2007. Revising how the computer program CERVUS accommodates genotyping error increases success in paternity assignment. Mol Ecol. 16:1099–1106.
Kempenaers B, Peters A, Foerster K. 2008. Sources of individual variation in plasma testosterone levels. Philos Trans R Soc B Biol Sci. 363:1711–1723.
Ketterson ED, Nolan V Jr. 1992. Hormones and life histories: an integrative approach. Am Nat. 140(suppl):S33–S62.
Ketterson ED, Nolan V Jr. 1994. Male parental behavior in birds. Annu Rev Ecol Syst. 25:601–628.
Ketterson ED, Nolan V Jr. 1999. Adaptation, exaptation, and constraint: a hormonal perspective. Am Nat. 154(suppl):S4–S25.
Ketterson ED, Nolan V Jr, Wolf L, Ziegenfus C. 1992. Testosterone and avian life histories: effects of experimentally elevated testosterone on behavior and correlates of fitness in the dark-eyed junco (*Junco hyemalis*). Am Nat. 140:980–999.
Kleven O, Jacobsen F, Izadnegahda R, Robertson RJ, Lifjeld JT. 2006. Male tail streamer length predicts fertilization success in the North American barn swallow (*Hirundo rustica erythrogaster*). Behav Ecol Sociobiol. 59:412–418.
Lessells CM, Boag PT. 1987. Unrepeatable repeatabilities: a common mistake. Auk. 104:116–121.
Lynn SE, Prince LE, Schook DM, Moore IT. 2009. Supplementary testosterone inhibits paternal care in a tropically breeding sparrow, *Zonotrichia capensis*. Physiol Biochem Zool. 82:699–708.
Lynn SE, Walker BG, Wingfield JC. 2005. A phylogenetically controlled test of hypotheses for behavioral insensitivity to testosterone in birds. Horm Behav. 47:170–177.
Magee SE, Neff BD, Knapp R. 2006. Plasma levels of androgens and cortisol in relation to breeding behavior in male bluegill sunfish, *Lepomis macrochirus*. Horm Behav. 49:598–609.
Magrath MJL, Komdeur J. 2003. Is male care compromised by additional mating opportunity? Trends Ecol Evol. 18:424–430.
McGlothlin JW, Jawor JM, Ketterson ED. 2007. Natural variation in a testosterone-mediated trade-off between mating effort and parental effort. Am Nat. 170:864–875.
McGraw K, Safran RJ, Evans MR, Wakamutsu K. 2004. European barn swallows use melanin pigments to color their feathers brown. Behav Ecol. 15:889–891.
Møller AP. 1994. Sexual selection and the barn swallow. Oxford: Oxford University Press.
Moore IT, Perfito N, Wada H, Sperry TS, Wingfield JC. 2002. Latitudinal variation in plasma testosterone levels in birds of the genus *Zonotrichia*. Gen Comp Endocrinol. 129:13–19.
Neff BD, Knapp R. 2009. Paternity, parental behavior and endogenous steroid hormone concentrations in nest-tending male bluegill. Horm Behav. 56:239–245.
Pinxten R, De Ridder E, Arckens L, Darras VM, Eens M. 2007. Plasma testosterone levels of male European starlings (*Sturnus vulgaris*) during the breeding cycle and in relation to song and paternal care. Behaviour. 144:393–410.
Primmer CR, Møller AP, Ellegren H. 1995. Resolving genetic relationships with microsatellite markers: testing system for the swallow *Hirundo rustica*. Mol Ecol. 4:493–498.
Rasbash J, Steele F, Browne W, Prosser B. 2004. A user's guide to MLwiN: version 2.0. London: centre for multilevel modeling.
Richardson DS, Jury FL, Blaakmeer K, Komdeur J, Burke T. 2001. Parentage assignment and extra-group paternity in a cooperative breeder: the Seychelles warbler (*Acrocephalus sechellensis*. Mol Ecol. 10:2263–2273.
Roff DA. 1992. The evolution of life-histories; theory and analyses. New York: Chapman and Hall.
Safran RJ, McGraw KJ. 2004. Plumage coloration, not length or symmetry of tail-streamers, is a sexually selected trait in North American barn swallows. Behav Ecol. 15:455–461.
Saino N, Møller AP. 1994. Secondary sexual characters, parasites and testosterone in the barn swallow, *Hirundo rustica*. Anim Behav. 48: 1325–1333.
Saino N, Møller AP. 1995. Testosterone-induced depression of male parental behavior in the barn swallow: female compensation and effects on seasonal fitness. Behav Ecol Sociobiol. 36:151–157.
Sasvári L, Péczely P, Hegyi Z. 2009. Plasma testosterone profile of male tawny owls *Strix aluco* in relation to breeding density, breeding experience, and offspring provision. Acta Ornithol. 44: 59–68.
Smorkatcheva AV, Bychenkova TN, Zavjalov EL. 2010. Parental responsiveness negatively correlates with fecal testosterone concentration in male mandarin voles (Mictrotus *mandarinus*). J Ethol. 28: 53–60.
Stearns SC. 1992. The evolution of life histories. Oxford: Oxford University Press.
Townsend DS, Moger WH. 1987. Plasma androgen levels during male parental care in a tropical frog (*Eleutherodactylus*). Horm Behav. 21: 93–99.
Townsend DS, Palmer B, Guillette LJ Jr. 1991. The lack of influence of exogenous testosterone on male parental behavior in a neotropical frog (*Eleutherodactylus*). Horm Behav. 25:313–322.
Trainor BC, Marler CA. 2001. Testosterone, parental behavior, and aggression in the monogamous California mouse (*Peromyscus californicus*). Horm Behav. 40:32–42.
Trumbo ST, Eggert AK. 1994. Beyond monogamy: territory quality influences sexual advertisement in male burying beetles. Anim Behav. 48:1043–1047.
Van Duyse E, Pinxten R, Eens M. 2000. Does testosterone affect the trade-off between investment in sexual/territorial behaviour and parental care in male great tits? Behaviour. 137:1503–1515.
Van Roo BL, Ketterson ED, Sharp PJ. 2004. Testosterone and prolactin in two songbirds that differ in paternal care: the blue-headed vireo and the red-eyed vireo. Horm Behav. 44:435–441.
Walsh PS, Metzger DA, Higuchi R. 1991. Chelex-100 as a medium for simple extraction of DNA for PCR-based typing from forensic material. Biotechniques. 10:506–513.
Williams TD. 2008. Individual variation in endocrine systems: moving beyond the "tyranny of the Golden Mean". Philos Trans R Soc B Biol Sci. 363:1687–1698.
Wingfield JC, Hegner RE, Dufty AM Jr, Ball GF. 1990. The "challenge hypothesis": theoretical implications for patterns of testosterone secretion, mating systems, and breeding strategies. Am Nat. 136: 829–846.
Wingfield JC, Hegner RE, Lewis D. 1991. Endogenous levels of luteinizing hormone and steroid hormones in relation to social status in the cooperatively breeding white-browed sparrow weaver, *Plocepasser mahali*. J Zool Soc Lond. 225:43–48.
Wynne-Edwards KE, Timonin ME. 2007. Paternal care in rodents: weakening support for hormonal regulation of the transition to behavioral fatherhood in rodent animal models of biparental care. Horm Behav. 52:114–121.